Biochemical Pharmacology, Vol. 35, No. 2, pp. 263–269, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

STEREOSELECTIVE BINDING OF 3-ACETOXY-, AND 3-HYDROXY-1,4-BENZODIAZEPINE-2-ONES TO HUMAN SERUM ALBUMIN

SELECTIVE ALLOSTERIC INTERACTION WITH WARFARIN ENANTIOMERS

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(*Received* 18 *April* 1985; *accepted* 16 *July* 1985)

Abstract—Stereoselective binding of oxazepam, lorazepam, temazepam and methyl lorazepam as well as of their acetates to human serum albumin was investigated by different techniques. The 2′-chlorine and the *N*(1)-methyl substitution exert opposite effects on the antipodes. Enantiomers of oxazepam acetate (OAc) and lorazepam acetate (LAc) displace diazepam. Allosteric interactions with warfarin were manifested by either mutually increased or decreased binding depending on the structure of benzodiazepine and on the configuration of both benzodiazepine and warfarin. The most remarkable effect could be observed in the simultaneous binding of (*S*)-lorazepam acetate and (*S*)-warfarin.

Although one pharmaceutical agent out of every four is marketed in racemic form, the specific action of chiral drug molecules is often manifested in stereospecificity [1]. Enantioselectivity has been established even for the binding to serum proteins [2] with large effects being observed for 3-substituted 1,4-benzodiazepines binding to human serum albumin (HSA) [3–6]. These investigations were performed by gel filtration and circular dichroism measurements and the origin of stereoselective binding preferring the (*S*)(+)-enantiomer was interpreted in terms of different conformations corresponding to the different absolute configurations [6].

In this work we studied how the substitution influences the binding of benzodiazepine enantiomers to HSA. Interaction studies with diazepam and warfarin serving as markers for specific binding sites on HSA [7] have also been performed. 3-Hydroxy- and 3-acetoxy-1,4-benzodiazepine-2-ones as well as their 2′-chlorine and *N*(1)-methyl derivatives (Table 1) have been investigated. Binding experiments using both racemates and resolved enantiomers were carried out by different methods including special ultrafiltration techniques based on stereoselective labelling [8, 9], quantitative evaluation of optical purities found in filtrate and retentate [10], as well as methods with HSA immobilized in microparticles [7] or coupled to Sepharose gel [11]. Although it was suggested several years ago that the binding of the 3-hydroxy compounds, oxazepam, lorazepam and temazepam might be stereoselective [12], the racemization of these compounds in aqueous solution [13] has not allowed experimental verification so far. Recently a peculiar elution profile was found for oxazepam in affinity chromatographic investigations suggesting the possibility of detecting stereoselective binding of the relatively short-lived enantiomers [14]. More detailed studies have been performed to test this hypothesis.

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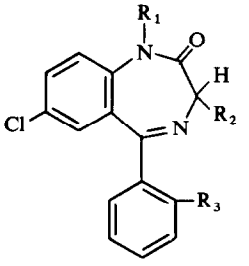
MATERIALS AND METHODS

Syntheses of racemic oxazepam, temazepam and their acetates [15], as well as 2-[^{14}C]oxazepam, 2-[^{14}C]OAc [16], 2-[^{14}C]oxazepam methylsuccinate [17], lorazepam and LAc [18] have been described. 2-[^{14}C]lorazepam, 2-[^{14}C]LAc and [^{3}H]diazepam were purchased from the Institute of Isotopes, Budapest. Me-Lorazepam and Me-lorazepam acetate (Me-LAc) as well as their 2-[^{14}C]labelled forms were synthesized according to ref. 19. 2-[^{14}C]temazepam and its acetate were prepared by the method of ref. 20. The radiolabelled and non-labelled OAc and LAc enantiomers were prepared by resolution of the racemates on a HSA-Sepharose 4B column; their chiroptical characterization was made by circular dichroism spectroscopy [10]. The radiochemical purity (>97%) of the enantiomers was checked by thin layer chromatography. The concentration of OAc and LAc solutions (containing 1 or 2% ethanol) was determined by u.v. spectroscopy (A^{230}_{OAc} : 3.4 × 10^4/M/cm; A^{232}_{LAc} : 3.5 × 10^4/M/cm). [^{14}C]Salicylic acid, tritiated water and [^{14}C]warfarin were purchased from The Radiochemical Centre (Amersham, U.K.). Na-warfarin and warfarin were obtained from Nyegaard and Co. (Oslo) and AB Ferrosan (Malmö), respectively. Racemic warfarin was resolved into its enantiomers as described [21].

Table 1. Structure of benzodiazepines investigated



R_1	R_2	R_3	
H	OH	H	Oxazepam
CH_3	OH	H	Temazepam
H	OH	Cl	Lorazepam
CH_3	OH	Cl	Me-Lorazepam
H	OAc	H	Oxazepam acetate (OAc)
CH_3	OAc	H	Temazepam acetate (TAc)
H	OAc	Cl	Lorazepam acetate (LAc)
Ch_3	OAc	Cl	Me-Lorazepam acetate (Me-LAc)

Ultrafiltration experiments were performed with Amicon YM10 membranes as described previously [8, 9], using non-fatty-acid-free HSA (Human Serum and Vaccine Institute, Budapest) and Ringer buffer (pH 7.4).

Binding studies with HSA immobilized in microparticles were done according to the procedure given by Sjöholm *et al.* [7], using HSA from AB KABI, Stockholm in 0.005 M phosphate buffer (pH 7.4) with 0.1 M KCl.

Binding studies by affinity chromatography were performed following the method of Lagercrantz *et al.* [11]; fatty acid free HSA (Sigma) coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) and 0.04 M phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.02% sodium azide were applied.

All binding experiments were performed at room temperature (23 ± 1°).

RESULTS

Stereoselective binding of oxazepam acetate and lorazepam acetate studied by different methods

(a) *Binding studies by ultrafiltration.* The stereoselective binding of OAc was studied by special ultrafiltration techniques [8, 9] performing stereoselective labelling of the racemate. The results were confirmed with resolved enantiomers. The binding characteristics were obtained from Scatchard plots and *nK* values are given in Table 2. The number of binding sites (*n*) was between 1 and 2; it could be determined with low accuracy.

(b) *Binding studies with HSA-microparticles.* The binding of OAc and LAc enantiomers was also studied with centrifugation using HSA immobilized in microparticles. This technique [7] requires only small amounts of material and loss of radioactivity due to non-specific binding appeared only at ligand concentrations below 10 μM. The binding data were analyzed by Scatchard representation and the *nK* values can be seen in Table 2. Similarly to the results

Table 2. Affinity (*nK*) and stereoselectivity obtained by different methods for the binding of OAc and LAc to HSA

Method	$C_{ligand} \times 10^5$ (M)	$C_{HSA} \times 10^5$ (M)	Oxazepam acetate			Lorazepam acetate		
			$n_R K_R$* (M^{-1})	$n_S K_S$* (M^{-1})	$\frac{n_S K_S}{n_R K_R}$	$n_R K_R$* (M^{-1})	$n_S K_S$* (M^{-1})	$\frac{n_S K_S}{n_R K_R}$
Ultrafiltration	0.1–7.3	2–10	1.1×10^4	5.5×10^4	5.0†	—	—	2.0‡
Microparticles	0.5–11.1	1.5	5.5×10^3	2.7×10^4	4.9	0.9×10^4	2.4×10^4	2.7
Affinity chromatography	7–20 nmol/sample	16.8	1.4×10^4	8.4×10^4	6.0	2.1×10^4	3.9×10^4	1.9

* Values relating to enantiomers.
† Cf. refs. 8–10.
‡ From ref. 10.

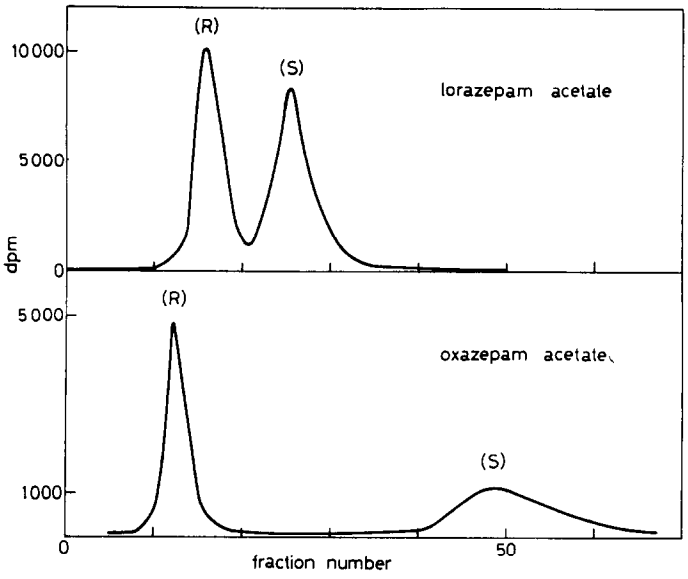


Fig. 1. Radiochromatogram of racemic [^{14}C]LAc (7 nmol, 19 mCi/mmol) and racemic [^{14}C]OAc (20 nmol, 1.6 mCi/mmol) on a HSA-Sepharose column. Elution was made by buffer. The volume of each fraction was 3.22 ml.

of ultrafiltration n values were between 1 and 2 and could be given with considerable uncertainty.

Binding studies by column affinity chromatography. The stereoselective binding of OAc and LAc to HSA was furthermore clearly shown by the resolution of racemates on column filled with immobilized HSA. Figure 1 shows the radiochromatogram of LAC and OAc racemates and indicates that the (*R*)-enantiomer of OAc is less strongly bound than that of LAc, while the opposite is true for the more tightly bound (*S*)-enantiomers. The method can also be used for an estimation of the association constants ΣnK from the elution volumes if certain column parameters are known [11]. In these experiments the elution volume was 15.3 ml for tritiated water and 85.3 ml for [^{14}C]salicylic acid. The volume of the gel was taken as 65% of the total (15 ml). The concentration of HSA in the gel was estimated from the binding of salicylic acid, using $nK = 4.25 \times 10^4\ M^{-1}$, a value obtained [22] for similar HSA concentration. Binding data are shown in Table 2.

(d) *Comparison of methods.* Binding affinity data obtained with different methods for the investigated pairs of enantiomers are collected in Table 2. As already mentioned Scatchard analysis of binding data obtained by ultrafiltration as well as by the microparticle technique indicated n values between 1 and 2 which are in accordance with the results found for oxazepam hemisuccinate [3]. However, owing to the poor solubility and low binding affinity of these ligands it was not possible to study higher saturations and to determine separate n and K values with reasonable accuracy (cf. [23]). The chromatographic technique is not informative in this respect, either. Thus, we chose the intercepts on the y-axis (nK) to characterize the binding strength. The correspondence of the nK values obtained by different methods under slightly different experimental conditions (quality and concentration of HSA, buffer composition) is within a threefold variation. The data for stereoselectivity (the ratio of respective nK values) are in better agreement. (*S*)-OAc has about 5–6 times higher affinity for HSA than (*R*)-OAc, while this factor is only 2–3 for LAc. The smaller stereoselectivity for LAc results from the stereospecific effect of the ortho-chlorine substitution which decreases the binding ability of the (*S*)-enantiomer as it was observed [24, 25] for benzodiazepines lacking a chiral centre, while increasing binding can be found in the case of the (*R*)-enantiomer (cf. Table 2).

Interactions in the binding of OAc and LAc enantiomers with diazepam and (RS)-warfarin as studied by microparticle technique

Displacement experiments were done in order to investigate the interaction of enantiomers with the specific diazepam and warfarin binding sites on HSA. Figures 2 and 3 show how the presence of OAc and LAc enantiomers influences the degree of binding of radioactive markers to HSA in microparticles. It can be seen (Figs. 2a and 3a) that the enantiomers displace diazepam, i.e. they increase its free concentration. The degree of displacement is in accordance with the relative values of association constants given in Table 2, with the effect of (*R*)-OAc being hardly measurable.

The interaction studies with racemic warfarin gave surprising results. Figure 2b shows that while (*R*)-LAc does not influence the binding of warfarin, (*S*)-LAc does markedly increase its binding. The effect of OAc enantiomers on warfarin binding (Fig. 3b) is not so striking, though the same phenomenon can be observed for (*S*)-OAc. The interaction between warfarin and (*S*)-LAc resulting in an increased binding is mutual, as can be seen in Fig. 4 showing the effect of warfarin on the binding of radioactive LAc

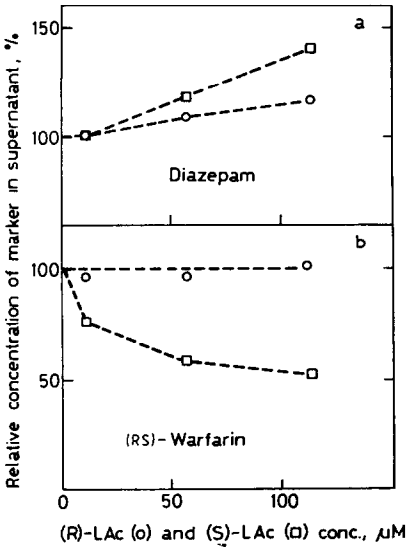


Fig. 2. Displacement of [^{3}H]diazepam (11.3 μM) and [^{14}C]-(*RS*)-warfarin (12.3 μM) from HSA in microparticles (14.1 μM) by (*S*)-LAc (□) and (*R*)-LAc (○); 46% of diazepam and 60% of warfarin were bound in the controls equivalent to $nK = 1.0 \times 10^5\,M^{-1}$ and $2.2 \times 10^5\,M^{-1}$, respectively. These values may somewhat differ from those of independent sets of experiment owing to a slight variation in protein concentration between samples from the same batch of microparticles (cf. caption to Fig. 3).

enantiomers. It is clear that Figs. 4 and 2b are counterparts.

Interaction of 3-hydroxy- and 3-acyloxy-benzodiazepines with warfarin enantiomers as studied by chromatographic method

The chromatographic technique is also useful for studying drug interactions through protein binding sites [11]. In these experiments radioactive benzo-

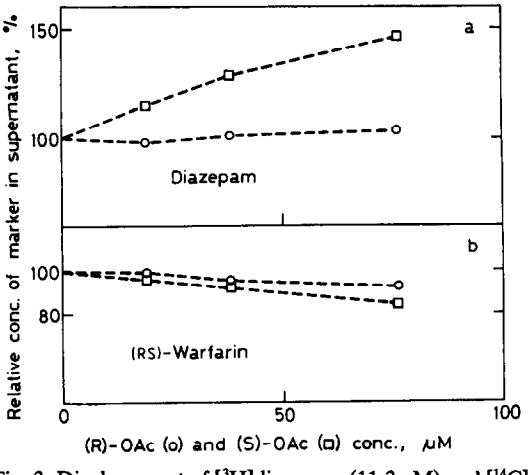


Fig. 3. Displacement of [^{3}H]diazepam (11.3 μM) and [^{14}C]-(*RS*)-warfarin (13.6 μM) from HSA in microparticles (14.1 μM) by (*S*)-OAc (□) and (*R*)-OAc (○); 53% diazepam and 62% warfarin were bound in the controls equivalent to $nK = 1.4 \times 10^5\,M^{-1}$ and $2.9 \times 10^5\,M^{-1}$, respectively (cf. caption to Fig. 2).

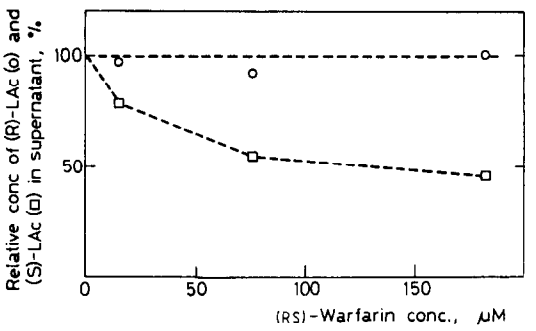


Fig. 4. Displacement of [^{14}C](*S*)-LAc (□, 11.6 μM) and [^{14}C](*R*)-LAc (○, 11.6 μM) from HSA in microparticles (15.5 μM) by (*RS*)-warfarin; 27% of (*S*)-LAc and 14% of (*R*)-LAc were bound in the controls.

diazepines were applied to the column in tracer amount (10 nmol) and the radiochromatograms were detected with eluents containing non-labelled (*R*)- or (*S*)-warfarin in 10^{-4} M concentration. Table 3 shows the influence of warfarin enantiomers on the elution volumes of the compounds investigated. As can be seen, in buffer eluent all compounds but lorazepam bind stereoselectively to HSA. Though Me-lorazepam could not be resolved either, its elution gave a broad peak suggesting slightly stereoselective binding beside racemization. The resolution of oxazepam and temazepam showing even higher stereoselectivities than the corresponding acetates is of principal interest, since in aqueous solution they are subject to racemization [13]. As already reported [14], oxazepam showed an irregular elution profile suggesting the racemization of desorbed molecules. Now the fast elution on a short, low capacity column allowed total separation. Enantiomeric stability is, however, confined to protein bound states as proved by the following experiment. Fractions corresponding to the two peaks obtained from temazepam were separately collected and the material recovered was applied again to the same column. Both samples gave chromatograms identical with that of the racemate.

Comparing oxazepam to temazepam as well as the corresponding acetates to each other it can be seen that the N(1)-methyl substitution enhances the stereoselectivity as a result of increased binding for the (*S*)- and decreased binding for the (*R*)-enantiomer. The presence of (*R*)-warfarin in the eluent does not change the elution volume of the first peaks (V_1) which correspond to (*R*)-enantiomers, and the binding of lorazepam and Me-lorazepam is also unchanged. There is a decrease, however, in the elution volumes of (*S*)-benzodiazepines (V_2) by the eluent containing (*R*)-warfarin. The only exception is (*S*)-LAc the binding of which is increased by (*R*)-warfarin. (*S*)-warfarin like its antipode does not influence the binding of (*R*)-benzodiazepines but its effect on the (*S*)-enantiomers is more discriminative. In cases of oxazepam, temazepam and TAc there are similar decreases like those found with (*R*)-warfarin, while the binding of Me-lorazepam and Me-LAc is almost unchanged. The oxazepam esters, however, show increased binding and (*S*)-LAc is practically stuck to the column. These results are in good correspondence with those found with the microparticle

Table 3. The effect of warfarin enantiomers on the elution volumes (V_1, V_2 in ml) of racemic [^{14}C]3-hydroxy- and [^{14}C]3-acyloxy-1,4-benzodiazepin-2-ones from HSA-Sepharose column (V_0 = 6.4 ml)

	Buffer		(*R*)-warfarin		(*S*)-warfarin	
Compound	V_1	V_2	V_1	V_2	V_1	V_2
Oxazepam	11	35	11	23	11	23
Temazepam	10	48	10	35	10	35
Lorazepam	13		12		12	28
Me-Lorazepam	18*		18*		18*	
Oxazepam acetate	12	31	12	28	12	67
Oxazepam methylsuccinate	13	39	12	31	12	48
Temazepam acetate	9	41	9	34	9	35
Lorazepam acetate	13	20	12	45	13	>130†
Me-Lorazepam acetate	11	28	11	26	11	24

* Broad peak of irregular shape.
† Half of the total radioactivity could be eluted only with 1% HSA solution.

Table 4. The drift of the second peak in the radiochromatogram of lorazepam on HSA-Sepharose column with warfarin eluent (10^{-4} M) of different enantiomeric composition

Composition of warfarin (%)		Fraction number	
(*R*)	(*S*)	1st peak	2nd peak
100	0	10	—
75	25	9	11
50	50	9	15
25	75	9	17
10	90	9	19
0	100	9	24

technique when racemic warfarin was used. The most intriguing is the interaction between (*S*)-warfarin and lorazepam. Approximately half of the radioactivity corresponding presumably to (*S*)-lorazepam shows significantly increased binding making an "apparent" resolution possible as can be seen in Fig. 5. The fore-shoulder of the second peak was reproducible. Table 4 shows that by varying the enantiomeric composition of the warfarin eluent a monotonous shift in the position of the second peak can be observed.

DISCUSSION

All 3-substituted benzodiazepines investigated but lorazepam showed stereoselective binding to HSA. It was determined by different methods that (*S*)-OAc has 5 to 6 and (*S*)-LAc 2 to 3 times higher binding affinities than their enantiomers. For the other compounds the measure of stereoselectivity can be evaluated from their affinity chromatographic separation. By this method it was possible even to resolve compounds subject to spontaneous racemization in aqueous solution, such as oxazepam and

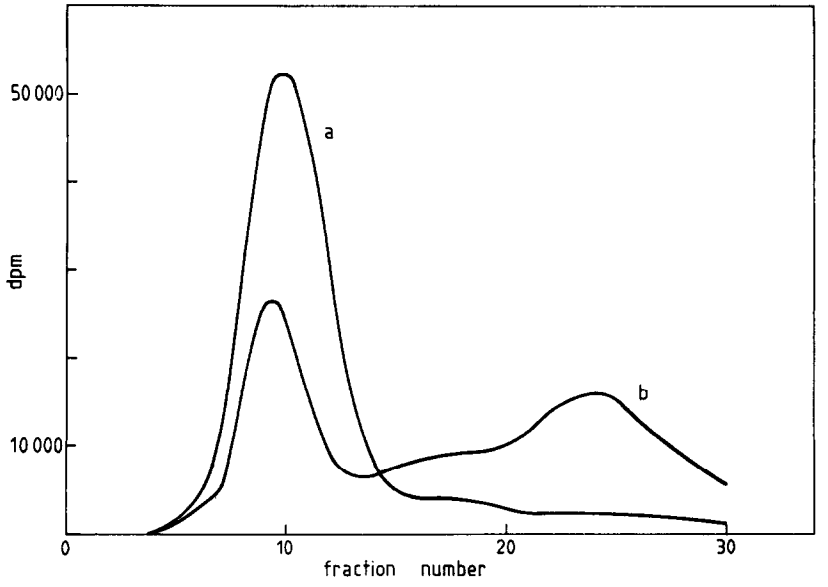


Fig. 5. Radiochromatogram of racemic [^{14}C]lorazepam on a Sepharose-HSA column (V_0 = 6.4 ml, $V_{fraction}$ = 1.27 ml). Elution was made by (a) 10^{-4} M (*R*)-warfarin, (b) 10^{-4} M (*S*)-warfarin.

temazepam. It proves that these 3-hydroxy compounds are enantiomerically stabile in their bound state and furthermore that they bind to HSA with even higher stereoselectivity than their acetates. Comparing oxazepam to lorazepam and to temazepam as well as the acetates to each other it was found that both the 2′-Cl and the *N*(1)-methyl substituents exert opposite influences on the binding of the enantiomers. The enantiospecific substituent effects of *N*(1)-methyl and 2′-Cl may be interpreted by either of two assumptions, as follows: (i) the enantiomers of the same benzodiazepine bind to separate sites on HSA; (ii) the enantiomers bind to the same site but in different conformation, just as they exist in solution. In view of the experiments showing that both enantiomers of OAc and LAc displace [^{3}H]diazepam from its specific binding site with an efficiency related to their binding strength (Figs. 2 and 3), the interaction is believed to be competitive favouring the second alternative. The enantioselective binding may therefore be accommodated with the concept of a specific benzodiazepine binding site preferring the conformation of (*S*)-enantiomers.

The interation of 3-hydroxy- and 3-acyloxy-benzodiazepines with (*S*)-warfarin is unexpected since diazepam was found to bind independently from warfarin [7] and bilirubin [26]. The interaction appeared only with (*S*)-benzodiazepine enantiomers and is manifested in both decreased and increased binding (Table 3). In view of the mutual enhancement (cf. Figs. 2b and 4) this phenomenon can be interpreted as an allosteric interaction between the bound ligands. The sign and the measure of the free energy coupling [27] vary with the structure of the interacting molecules.

The enhancement of binding seems to be strongly dependent on the presence of the ortho-chlorine substituent in the detached phenyl ring and, to some extent, on the nature of the 3-substituent of the 1,4-benzodiazepine ring. The *N*(1)-methyl substituent prevents the phenomenon. (*R*)-Warfarin showed increased binding only with (*S*)-LAc but even this effect was much weaker than that provoked by the (*S*)-stereoisomer. The different behaviour of warfarin enantiomers may be explained by the suggestion [28] that the orientation of the 4-hydroxycoumarin nucleus would be different for the antipodes when bound to HSA. A stoichiometric description of racemic warfarin binding to HSA [29] was possible with two main binding constants. Beside proving a weak stereoselectivity in warfarin binding [11, 30], affinity chromatographic experiments have shown that the enantiomers partly displace each other [11]. Accordingly, the specific interaction found between (*S*)-lorazepam and (*S*)-warfarin (Table 4) was considerably inhibited by the presence of even 10% of (*R*)-warfarin, suggesting a common binding site shared by both warfarin enantiomers.

Much work has been done to detect and characterize specific binding sites or areas on HSA [31, 32]. On the other hand, Brodersen *et al.* [33, 34] emphasized that the site-oriented description is an oversimplification and presented examples when drug binding, especially the quantitative treatment of multiple binding and cobinding can be more correctly described with a stoichiometric analysis without assuming preformed binding sites. The main conclusions of our work are based on experiments using low drug/protein concentration ratios which relate to the binding process of the highest affinity. The sensitive alteration in the binding behaviour brought about by small structural variations in the benzodiazepine enantiomers supports the existence of a benzodiazepine binding site preferring the (*S*)-enantiomers. However, these results prove that separate binding sites on HSA are not independent even if certain ligands (e.g. diazepam) can be used as specific markers. What is true for diazepam is, however, not necessarily applicable to all benzodiazepines. Some members of this group of drugs even weakly bound to flexible HSA (i.e. lorazepam and (*S*)-LAc) can provoke unexpected non-competitive interactions with (*S*)-warfarin and (*R*)-warfarin, hence considerations on the independent character of warfarin and "benzodiazepine" binding sites can be hazardous. Another main conclusion of this work is that the investigation of the enantiomers instead of the racemate of chiral drugs is unavoidable in order to get meaningful results relevant on the molecular level.

Acknowledgements—Financial support from the Hungarian Academy of Sciences, the Swedish Medical Research Council, I.F.: a Foundation for Pharmaceutical Research and Magnus Bergvalls stiftelse are acknowledged. The authors are indebted to Dr A. Gelléri for synthesizing temazepam acetate, to Mrs E. Simon-Trompler for samples of lorazepam and lorazepam acetate and to Mrs A. Kiss and Mrs E. Tidare for skilful technical assistance. Information on unpublished results by Prof. R. Brodersen is gratefully acknowledged.

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